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INCREASE OF ETHANOL PRODUCTIVITY BY CELL-RECYCLE FERMENTATION OF FLOCCULATING YEAST

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Using the recombinant flocculating Angel yeast F6, long-term repeated batch fermentation for ethanol production was performed and a high volumetric productivity resulted from half cells not washed and the optimum opportunity of residual glucose 20 g l^{-1} of last medium. The obtained highest productivity was 2.07 g l^{-1} h^{-1}, which was improved by 75.4% compared with that of 1.18 g l^{-1} h^{-1} in the first batch fermentation. The ethanol concentration reached 8.4% corresponding to the yield of 0.46 g g^{-1}. These results will contribute greatly to the industrial production of fuel ethanol using the commercial method with the flocculating yeast.

Since the oil crisis of 1973, the biological production of ethanol has become one of the top candidates for the replacement of petrochemicals for energy usage. At the same time the use of bioethanol is considered advantageous from an environmental point of view. However feasible solutions do not guarantee a breakthrough in the usage of the renewable resource unless the prices of products obtained are competitive with those from fossil raw materials. Numerous studies have been performed dealing with various aspects of ethanol fermentation. The raw material represents a major share of the production costs [1], and any improvement ethanol productivity should lead to an improved process economy.

Long-term cell-recycle fermentation showed a higher volumetric productivity of ethanol than a simple batch fermentation, because a shorter fermentation time and higher substrate consumption rates were obtained in cell-recycle fermentation. However, centrifugation is usually too complex to be economically viable on a large scale and is difficult to maintain aseptic conditions. The ability of yeast cells to flocculate is of considerable importance for the fuel ethanol industry, as it provides an effective, environment-friendly, simple and cost-free way to separate yeast cells from the final culture fluid, thus facilitating further downstream processing.

It also is one of the feasible approaches that the production of byproducts should be minimized. Quantitatively, carbon dioxide, glycerol and cell biomass are the most significant byproducts during anaerobic ethanol production by *Sacharomyces cerevisiae* [2]. Carbon dioxide is an inevitable fermentation product, but the off-gas can be sold as a high-quality raw material. Substantial amount of glycerol, up to 10% (w/w) of the carbon source, may be formed under anaerobic conditions for the purpose of reoxidising surplus NADH being formed in anabolism [3–5]. Another research states that glycerol consumes up to 4% of the carbon source in industrial fermentations [6]. Glycerol is also produced as a compatible solute during osmotic stress [7].

Using the flocculating Angel yeast F6 which was described previously [8], ethanol was attempted to be produced at a high volumetric productivity by cell-recycle fermentation. Although many studies have constructed the flocculation yeast [9–12], the recombinant yeast strain has seldom been applied to ethanol production by cell-recycle fermentation. Therefore, the flocculation Angel yeast F6 was recycled without centrifugation and the experiment performed for 16 recycle rounds and a highest productivity of 2.07 g l^{-1} h^{-1} during 8th recycle round was achieved.

MATERIALS AND METHODS

Microorganism and media. Angel yeast F6 was constructed previously and maintained in a frozen state at –70°C. The preculture contained (g l^{-1}): yeast extract – 8.5, glucose – 30.0, $(NH_4)_2SO_4$ – 0.5, $MgSO_4$ – 0.1 and $CaCl_2$ – 0.06. The fermentation medium consisted of (g l^{-1}): yeast extract – 5.0, glucose – 150.0, $(NH_4)_2SO_4$ – 0.5, $MgSO_4$ – 0.65, $CaCl_2$ – 2.0 and KH_2PO_4 – 1.5 in static Erlenmeyer flasks (250 ml) containing 150 ml. Five percent (v/v) of the seed culture was inoculated, and the initial pH of the medium was adjusted to 5.0.

Batch fermentation. Growing cells of *S. cerevisiae* Angel yeast F6 were precultured in a 250 ml Erlenmeyer flasks containing 50 ml of the growth medium at 30°C with agitation at 200 rpm for 12 h. This seed culture was then inoculated into a 250 ml Erlenmeyer flasks containing 150 ml of the fermentation medium. The initial pH was adjusted to 5.0, although pH was not controlled dur-

Table 1. Choice of re-inoculated opportunity

Indicators	RG of last medium, g/1									
	40		30		20		10		0	
Fermentation time, h	48	56	48	56	48	56	48	56	48	56
EC, %, v/v	6.2	7.0	7.1	7.4	7.7	7.7	6.6	6,6	6.7	6.7
Yield, g/g	0.34	0.40	0.41	0.42	0.44	0.44	0.38	0.38	0.38	0.38

ing the fermentation. Temperature was maintained at 30°C and the fermentation was performed statically.

Cell-recycle fermentation. After the completion of batch fermentation with a working volume of 150 ml in a 250 ml Erlenmeyer flasks, cell-recycled fermentation in the Erlenmeyer flasks was performed. Whenever the glucose concentration fell below about 20 g l^{-1}, the cultural medium was transferred and half of cells were kept. A fresh medium was added to the cells recovered from the Erlenmeyer flasks to give 150 ml in all recycle rounds. Cell-recycle fermentation with the flocculating cell was performed for 16 recycle rounds.

Analytical methods. *Glucose concentration assay*. Glucose concentration was measured by using biosensor analyzer with a glucose oxidase (GOD) coupled enzymatic membrane.

Dry cell weight assay. Dry cell weight (DCW) was obtained after centrifuging at 1500 g for 15 min, washed twice with distilled water, re-centrifuged and dried at 105°C for 24 h.

Ethanol concentration assay. Ethanol concentration was measured by using Shimadzu GC-2010 gas chromatography (Japan) with a PEG20M column (internal diameter 0.25 mm, length 3 m) and a FID detector. Nitrogen was used as the carrier gas at a flow rate of 3 ml/min while n-propanol was used as the internal standard. The column temperature was maintained at 220°C.

RESULTS AND DISCUSSION

Choice of the cell-recycle opportunity. It was proved in the experiment that glucose was the most important factor influencing the ethanol production of the recombinant Angel yeast F6 (data not shown). The residual glucose concentration of the medium was taken as the standard of the choice of the cell-recycle opportunity. As was shown in Table 1, the yeast cells were reused when the residual glucose (RG) of last medium was more than 30 g l^{-1}, the fermentation time was longer than those of the other three ways. The cells were recycled when the residual glucose of last medium decreased to less than 10 g l^{-1}, the ethanol concentrations in the fermentation media were lower and fermetation time were longer. The optimum opportunity for yeast cells to reuse was the residual glucose concentration of 20 g l^{-1} in the fermentation medium with the maximum ethanol concentration of 7.7% and yield of 0.44 g g^{-1} obtained.

Repeated batch fermentation with all and half cells. Repeated batch fermentation means that culture broth is centrifuged at the end of ordinary batch fermentation and centrifuged cells are recycled for the next batch fermentation [13]. In this study, the recombinant Angel yeast F6 flocculated from the early stationary growth phase. At the end of batch fermentation yeast cells sedimented at the bottom of flask and was reused for the next batch by taking the above medium out, without centrifugation.

The effects of the different amount of recycled cells on the ethanol production were investigated. As shown in Fig. 1, the variety of biomass was especially obvious and increased with the rounds of cell-recycle fermentation. The final biomass of the sixth batch fermentation with half recycled cells increased to 4.22 g l^{-1}, which was 1.7-fold compared with the 2.48 g l^{-1} of the first original batch. However, when repeated batch fermentation performed with all cells to reuse, the final biomass was 5.94 g l^{-1} of the sixth batch fermentation, which was 2.4-fold compared with the 2.48 g l^{-1}. The ethanol concentration also increased with the rounds of cell-recycle fermentation during the six batch fermentation. But the variety of the ethanol concentration was only improved by 18.3% from 7.1 to 8.4%, not so much as the biomass. In the second repeated batches the ethanol concentration of fermentation with all recycled cells was 7.6%, higher than those with half recycled cells, which was 7.2%. Then they were maintained similar after the third repeated recirculation.

The ethanol concentration could be improved in the fermentation with cell recycled, but had not obvious difference between the fermentations with all and half recycled cells. The repeated fermentation with half recycled cells could reduce the quantity of cells, so one of the most significant byproducts during anaerobic ethanol production was reduced.

Repeated batch fermentation with recycled cells washed and not washed. The influence of washing reused cells on ethanol formation was investigated. Experiments were divided into two groups. In one group (A) recycled cells were washed with distilled water before reuse during six batch fermentation. In the other group (B) recycled

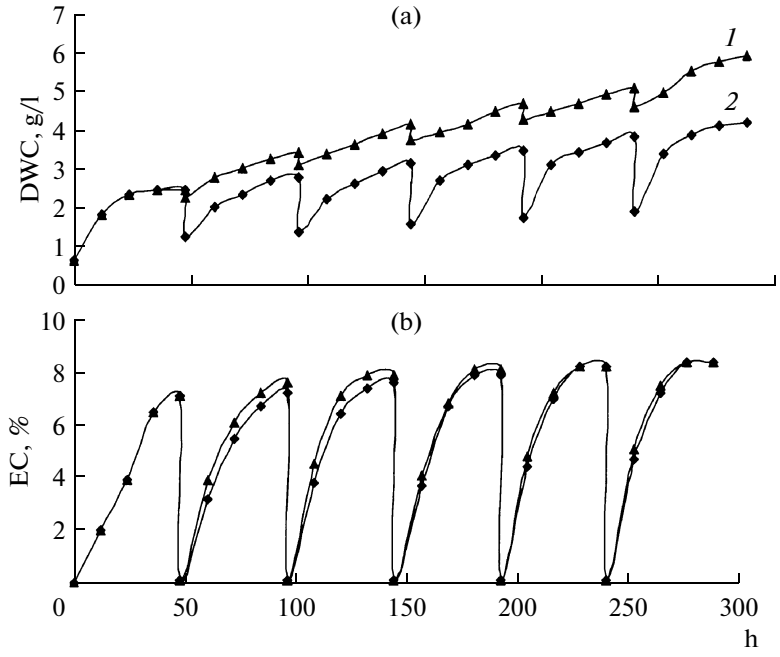

Fig. 1. Time courses of biomass (a) and ethanol (b) concentration in repeated batch fermentation with all (1) and half recycled cells (2).

cells without washing were directly reused. The results were shown in Tables 2 and 3, respectively.

As shown in the two tables, half cells were reused for the next batch fermentation. The final dry cell weight (DCW) in each cycle increased with the rounds of fermentation. The biomass of group A was similar to that of group B. But the ethanol concentration of group A increased from 7.1 to 7.6%, and yield was from 0.39 to 0.42 g g^{-1}. Both were lower than those in group B, which were from 7.1 to 8.1%, and from 0.39 to 0.45 g g^{-1}. Furthermore the highest productivity of 1.90 g l^{-1} h^{-1} in group B was higher than that of 1.79 g l^{-1} h^{-1} in group A. Fermentation with recycled cells not washed was prior to that with recycled cells washed, which indicated that the activity of washed cells were decreased and the ability of ethanol formation was inhibited.

Repeated batch fermentation with the flocculating Angel yeast F6. Time courses of biomass, glucose and ethanol concentration in 16 rounds of repeated fermentation with half recycled cells not washed were shown in Fig. 2. The biomass increased with the increase of recycles constantly during the first 11 batches and reached 5.21 g l^{-1} at the end of 11th batch, which was 2.1 times as the first ordinary batch 2.48 g l^{-1}. The final ethanol concentration kept increasing in the first 10 batches and reached 8.4% at the end of the 10th batch. Although the final ethanol concentration did not change much from 6.5 to 8.4%, the fermentation time varied from 32 h to 48 h, and the volumetric productivity varied from 1.08 to 2.07 g l^{-1} h^{-1}. In the 8th batch, the final ethanol concentration was 8.3% for 32 h, and corresponded to an ethanol yield of 0.46 g g^{-1} and a volumetric productivity of 2.07 g l^{-1} h^{-1}, which was improved by 75.4% compared with that of 1.18 g l^{-1} h^{-1} in the first batch fermentation.

Table 2. Repeated batch fermentation with recycled cells washed

Indicators	Round of fermentation					
	1	2	3	4	5	6
Initial DCW, g/l	0.65	1.22	1.45	1.62	1.69	1.81
Final DCW, g/l	2.48	2.88	3.23	3.34	3.52	3.64
Fermentation time, h	**48**	**46**	**42**	**38**	**36**	**34**
EC, %, v/v	7.1	7.0	7.2	7.4	7.5	7.6
Yield, g/g	0.39	0.39	0.40	0.41	0.41	0.42
Productivity, g/l h	1.18	1.22	1.37	1.56	1.66	1.79

Table 3. Repeated batch fermentation with recycled cells not washed

Indicators	Round of fermentation					
	1	2	3	4	5	6
Initial DCW, g/l	0.65	1.24	1.39	1.52	1.71	1.84
Final DCW, g/l	2.48	2.78	3.11	3.41	3.78	3.95
Fermentation time, h	**48**	**46**	**42**	**38**	**36**	**34**
EC, %, v/v	7.1	7.2	7.4	7.6	7.9	8.1
Yield, g/g	0.39	0.40	0.41	0.42	0.44	0.45
Productivity, g/l h	1.18	1.25	1.41	1.60	1.76	1.90

Actually the recycled fermentation with the recombinant flocculating Angel yeast F6 has been performed for 16 rounds (Fig. 2). From the 6th to 12nd rounds, the final ethanol concentration kept stability of more than 8.0% and each fermentation time did not exceed 48 h of the ordinary batch fermentation. During the 13rd to 16th batches, the ethanol concentrations varied from 7.9 to 6.5%. As the volumetic productivity declined 1.08 g l^{-1} h^{-1} in the 16th round, which was lower than that of the ordinary batch fermentation, it was not worthy to continue the cell-recycle fermentation and 15 rounds of repeated-batch fermentation were accessible.

Microorganisms were found to actively produce metabolites under anaerobic conditions. Repeated fermentation using the flocculation yeast has been performed

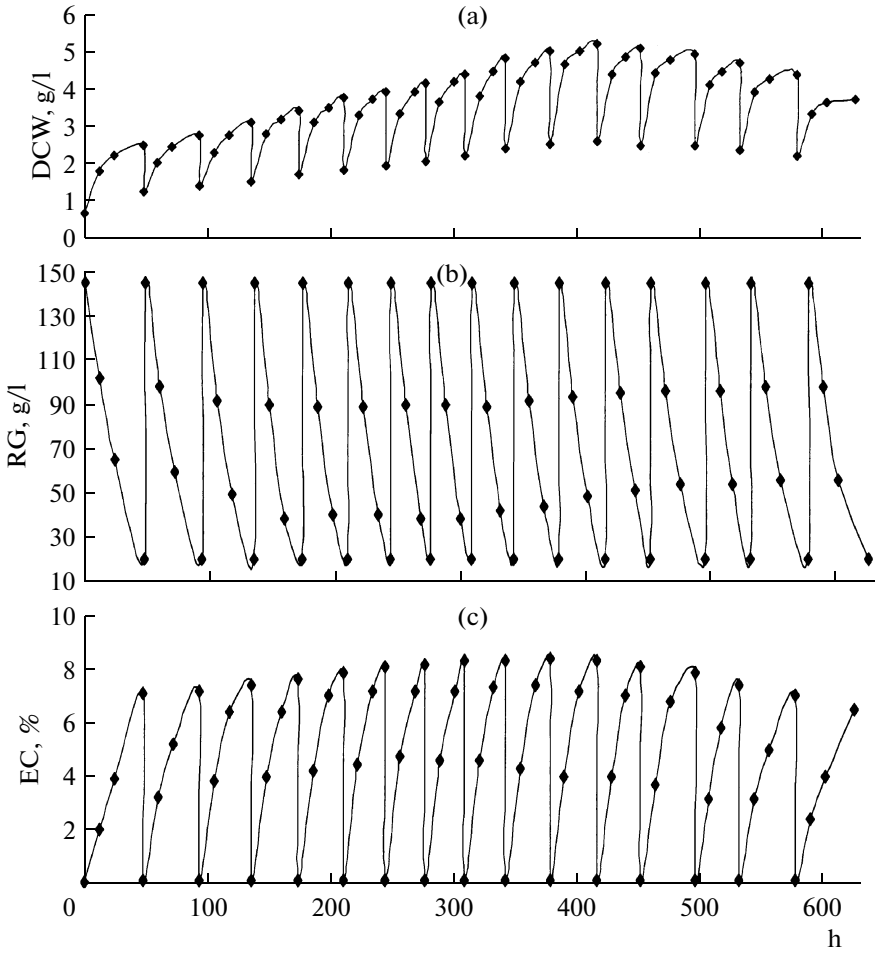

Fig. 2. Time courses of repeated fermentation with recycled cells of recombinant Angel yeast F6: a – DCV, g/l, b – RG, g/l; c – EC, %.

under anaerobic condition and high cell mass has been easily obtained, resulting in much higher productivity.

* * *

It was the optimum opportunity for yeast cells of the recombinant Angel yeast F6 to reuse that the residual glucose of the medium decreased to 20 g l^{-1}. The ethanol yield and concentration could be improved in the fermentation with cell recycle, but had not obvious difference between the fermentation with all and half recycled cells. Fermentation with recycled cells not washed was prior to that with recycled cells washed, which indicated that the activity of washed cells decreased and the ability of ethanol formation was inhibited.

Long-term repeated batch fermentation for ethanol production with the recombinant flocculating Angel yeast F6 was performed and a high volumetric productivity resulted from half cells not washed and the optimum opportunity of RG 20 g l^{-1} of last medium. The obtained highest productivity was 2.07 g l^{-1} h^{-1}, which was improved by 75.4%, compared with that of 1.18 g l^{-1} h^{-1} in the first batch fermentation. The ethanol concentration reached 8.4% corresponding to the yield of 0.46 g g^{-1}. These results will contribute greatly to the industrial production of fuel ethanol using the commercial method with the constructed flocculating yeast.

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